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Shifting *Nicotiana attenuata*'s diurnal rhythm does not alter its resistance to the specialist herbivore *Manduca sexta*

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Research Article

Abstract *Arabidopsis thaliana* plants are less resistant to attack by the generalist lepidopteran herbivore *Trichoplusia ni* when plants and herbivores are entrained to opposite, versus identical diurnal cycles and tested under constant conditions. This effect is associated with circadian fluctuations in levels of jasmonic acid, the transcription factor MYC2, and glucosinolate contents in leaves. We tested whether a similar effect could be observed in a different plant–herbivore system: the wild tobacco *Nicotiana attenuata* and its co-evolved specialist herbivore, *Manduca sexta*. We measured larval growth on plants under both constant and diurnal conditions following identical or opposite entrainment, profiled the metabolome of attacked leaf tissue, quantified specific metabolites known to reduce *M. sexta* growth, and monitored *M. sexta* feeding activity under all experimental conditions. Entrainment did not consistently affect *M. sexta* growth or plant defense induction. However, both were reduced under constant dark conditions, as was *M. sexta* feeding activity. Our data indicate that the response induced by *M. sexta* in *N. attenuata* is robust to diurnal cues and independent of plant or herbivore entrainment. We propose that while the patterns of constitutive or general damage-induced defense may undergo circadian fluctuation, the orchestration of specific induced responses is more complex.

Keywords: Circadian clock; diurnal rhythm; *Manduca sexta*; *Nicotiana attenuate*; plant–herbivore interactions
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INTRODUCTION

Through the Earth's rotation, day and night alternate in an approximately 24 h diurnal cycle. Most organisms have developed an internal circadian clock in order to synchronize their activities with predictable diurnal events (reviewed in Bell-Pedersen et al. 2005). Plants have been shown to gain a fitness advantage from having a circadian clock synchronized with their external environment (reviewed in Yerushalmi and Green 2009). Furthermore, some plant responses to abiotic stress (reviewed in Sanchez et al. 2011) and to biotrophic pathogen attack (Bhardwaj et al. 2011; Wang et al. 2011; Zhang et al. 2013) are mediated by the circadian clock, but less is known about plant responses to herbivore attack.

Goodspeed et al. (2012) showed that larvae of the generalist lepidopteran *Trichoplusia ni* have a circadian feeding pattern when observed on artificial diet. They found that *T. ni* entrained to a 12 h:12 h light: dark (LD) cycle grew larger and caused more damage on *Arabidopsis thaliana* plants that had been entrained to a diurnal regime opposite to that of larvae (out-of-phase), versus when plants and larvae were entrained to the same regime (in-phase). These experiments were performed under constant dark (DD) or constant light (LL) conditions, setups classically used in order to separate circadian from diurnal phenomena (Harmer 2009). Similar effects could be demonstrated for harvested fruits and vegetables from several plant families entrained under 12 h:12 h LD cycles prior to, or during infestation by *T. ni* (Goodspeed et al. 2013b). As had been shown for pathogen attack (Bhardwaj et al. 2011; Wang et al. 2011; Zhang et al. 2013), Goodspeed et al. (2012, 2013a) furthermore showed that in-phase plants of arrhythmic *A. thaliana* mutant lines (*CCA1-OX* and the *LUX ARRHYTHMO* knockout *lux2*) were not better defended than out-of-phase plants of the same lines under DD or LL conditions, implicating the clock in the anticipation of defense to *T. ni*. Additionally, jasmonic acid (JA) and salicylic acid (SA) levels fluctuated constitutively in *A. thaliana* plants under DD and LL, in oppositely phased circadian cycles, and *A. thaliana* lines deficient in jasmonate biosynthesis (*allene oxide synthase* (*aos*), *jasmonate resistant 1*(*jar1*) were not better defended when entrained in-phase with *T. ni* (Goodspeed et al. 2012). This consistent effect of

various plant clocks on the rhythmically feeding *T. ni* is intriguing, albeit difficult to understand in an evolutionary context, given that *T. ni* has rarely been observed feeding on *A. thaliana* in nature (Jander 2012).

In a follow-up study, Goodspeed and colleagues (2013b) demonstrated circadian fluctuation of glucosinolate contents in *A. thaliana* leaves as well as in harvested cabbage, with glucosinolate contents peaking during the day at levels threefold the night-time trough. These fluctuations had a similar phase to the constitutive JA fluctuations demonstrated in the 2012 study and were proposed as a direct, if not complete, explanation of synchronized plants' superior resistance to *T. ni*, since this effect is likely to be mediated by JA-induced metabolites and not directly by JA (but see Kallenbach et al. 2012). However, the *A. thaliana myb28myb29* mutant, which does not produce aliphatic glucosinolates, also displayed greater resistance to *T. ni* after co-entrainment of plants and larvae, although overall resistance of *myb28myb29* was reduced in comparison to WT.

Thus, the most straightforward interpretation of the studies by Goodspeed and colleagues is that circadian fluctuation in JA-mediated defenses allows plants to anticipate herbivore attack. However there are other interpretations, and the "anticipatory defense" hypothesis suffers from some weaknesses: unlike foliar pathogens, herbivores do not depend on a circadian cycle of stomatal opening for attack. Even herbivores from the same family, sharing the same host plant, and sensitive to overlapping suites of jasmonate-mediated defenses may attack at different times of day, although individual herbivores do have characteristic activity patterns; and herbivore population dynamics are unpredictable (Wigglesworth 1972; Denno et al. 1995; Jander 2012).

We thus asked whether anticipatory defense can be observed in other plant–herbivore pairs (Jander 2012). Specifically, we tested whether the resistance of the wild tobacco *Nicotiana attenuata* Torr. ex. Watson (Solanaceae) to its specialist herbivore *Manduca sexta* Linne (Sphingidae) is also reduced when plants and herbivores are entrained out-of-phase with each other. *M. sexta* is both among the most damaging herbivores of *N. attenuata*, as well as an important pollinator (Kessler 2012). The intertwined evolutionary history of these two organisms should have afforded the opportunity for *N. attenuata* to anticipate attack by *M. sexta* larvae using any robust and expedient means.

We tested the hypothesis that out-of-phase entrainment of *N. attenuata* increases *M. sexta* growth and decreases *N. attenuata*'s elicitation of defense compounds under constant or diurnal experimental conditions. In designing our experiments, we considered that constant conditions can have detrimental effects on plant physiology: physiological disorders and photodamage in constant light (reviewed in Sysoeva et al. 2010), or carbon starvation in constant dark (e.g., Graf et al. 2010). In order to demonstrate a direct mechanism for the observed growth of *M. sexta* on differently entrained plants, we analyzed leaf material for general metabolomic fingerprints as well as target specialized metabolites before and after *M. sexta* feeding for all experimental set-ups, rather than analyzing levels of defense signaling hormones. *N. attenuata* accumulates a panoply of defense compounds in shoot tissue in response to *M. sexta* feeding, which have well-characterized effects on *M. sexta* growth rates. These include hydroxygeranyllinalool-diterpene glycosides (HGL-DTGs, Heiling et al. 2010), phenolics and putrescine conjugates (Kaur et al. 2010), and trypsin protease inhibitors (Zavala and Baldwin 2004). Although *M. sexta* is tolerant to high levels of nicotine, its growth is also reduced on nicotine-containing compared to nicotine-deficient plants, likely due to the metabolic cost of detoxification for larvae (Steppuhn et al. 2004).

We observed no difference in either *M. sexta* growth or *N. attenuata*'s elicitation of defense compounds as a result of synchronous versus asynchronous entrainment of plants and larvae under diurnal (LD) conditions; a small increase in production of defense compounds by asynchronously entrained plants and no difference in *M. sexta* growth under constant light (LL); and a transient increase in *M. sexta* growth coupled to a reduction in levels of defense metabolites, which already tended to be low, for asynchronously entrained pairs under constant dark (DD). We conclude that, while placing plants under constant conditions – especially DD – stresses plants and alters their capacity to mount resistance, there is no effect of in-phase versus out-of-phase entrainment of plants and herbivores on *N. attenuata*'s ability to resist *M. sexta* attack.

RESULTS

Growth of *M. sexta* larvae

We entrained plants under one of two regimes: in-phase or out-of-phase, infested plants with *M. sexta* larvae, which had been entrained under the in-phase regime, and measured larval growth under diurnal (LD) or constant conditions (LL, DD; $n = 26–45$ larvae, Figure 1). Under all three conditions, growth of *M. sexta* larvae (Figure 2) was similar on in-phase and out-of-phase plants ($P > 0.13$, statistical test results in Table S1). There was only a single measurement, day 7 under DD, for which larvae feeding on out-of-phase plants were significantly heavier than larvae on in-phase plants ($P = 0.0293$). However, overall growth rates of larvae were reduced under DD, as was mortality at the end of the 9 d experiment (DD, 27% mortality; LL, 43%; LD, 37%; DD vs. LL, $P = 0.038$; DD vs. LD, $P = 0.300$, Bonferroni-corrected G-tests of independence). Mortality did not differ between larvae on in- and out-of-phase plants (DD, 33.3% and 20.0%, $P = 0.2870$; under LL, 48.9% and 37.8%, $P = 0.1510$; under LD, 41.7% and 31.7%, $P = 0.2734$). While larvae also developed through instars at similar rates on in-phase and out-of-phase plants, development through instars was slower under DD when compared to LL (day 9: DD, mode: end of 3rd to beginning of 4th instar; LL: middle to end of 4th instar).

Untargeted metabolomic fingerprints of in-phase and out-of-phase plants

Metabolomic fingerprints measured in positive electrospray mode, which captures the majority of known induced metabolites in *N. attenuata* (Gaquerel et al. 2010; Kim et al. 2011), were similar for in-phase and out-of-phase rosette-stage *N. attenuata* plants after 3 d of herbivory by *M. sexta* larvae under LL, DD, and LD ($n = 5$ plants). We used both principal component analysis (PCA, Figures 3, S1) and volcano plots (Figure S2, File S1) to visualize the differences between sample groups. Metabolomic fingerprints of in-phase and out-of-phase plants in PCAs differed before herbivory in LL and LD, but did not differ after 3 d of herbivory, as revealed by

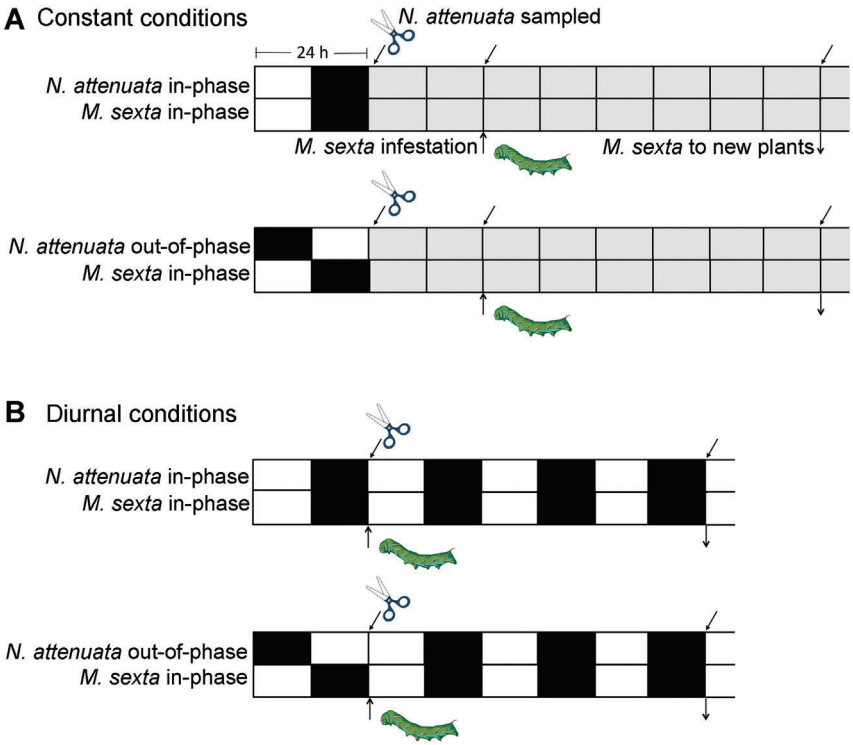

Figure 1. Experimental designs for assaying entrainment effects under constant or diurnal conditions
Plants and eggs were transferred from their respective entrainment regime to constant conditions 1 d before infestation (**A**), or for diurnal conditions (**B**), out-of-phase plants were transferred to the light/dark regime under which in-phase plants and larvae had been entrained, and infestation followed immediately. *Manduca sexta* infestation lasted for 3 d, after which larvae were placed on new, entrained plants of the same treatment group. After day 3, transfer to new plants occurred every 2 d due to increased larval feeding damage (not depicted). Boxes represent 12 h intervals of either light (white squares) or dark (black squares); the change from diurnal to constant conditions (constant light, LL or constant dark, DD) is indicated by the change to grey panels. *M. sexta* larvae always received the in-phase entrainment regime while *Nicotiana attenuata* plants received in-phase or out-of-phase entrainment regimes. Angled arrows from above show time points at which leaf tissue was collected (before transfer to constant conditions, before and 3 d after infestation by *M. sexta* larvae); arrows from below show when *M. sexta* larvae were transferred to or from plants. Plants were grown in their respective entrainment regimes for at least 10 d, while *M. sexta* were entrained as eggs and neonates for at least 1 d before infestation of plants (larvae hatched within the last 12 h before infestation).

overlapping confidence intervals for the sample groups; similarly, overlap was greater in DD after 3 d of herbivory (Figure 3). The explanatory value of PCA axes for the separation of sample groups is difficult to compare between individual PCAs, but separation in metabolomics fingerprints before and after herbivory was more pronounced in LL and LD than in DD, indicating a stronger response of plants to herbivory in LL and LD.

Transfer to constant conditions (LL and DD) itself had a strong effect on plant metabolic fingerprints: adding plant samples taken before a 1 d pre-infestation period (see methods) to PCAs for LL and DD shows that in-phase (12 h light:12 h dark) and out-of-phase plants (12 h dark:12 h light) in fact became less similar after 1 d of exposure to constant conditions (Figure S1). Only a small number of m/z features (20) had a more than two-fold change and a P-value < 0.05 in a comparison of in-phase and out-of-phase plants after 3 d herbivory in LL. Similarly, only 14 m/z features showed significant differences between in-phase and out-of-phase plants after 3 d herbivory in LD, while 66 m/z features showed significant differences between in-phase and out-of-phase plants after 3 d herbivory in DD (Figure S2). Hyponasty and strong reductions in starch contents were observed for plants in DD (Figure S3), indicating that stress responses to DD might have modified the induced defense response.

Quantification of target specialized metabolites

Because separation along PC1 in PCAs (Figure 3) likely reflects changes to both general (primary) and specialized metabolism induced by herbivory (e.g., Schwachtje and Baldwin 2008; Machado et al. 2013), we chose to quantify a number of well-characterized defense metabolites for which an effect on *M. sexta* growth had been shown in previous studies: nicotine (e.g., Steppuhn et al. 2004), 17-hydroxygeranyllinalool diterpene glycosides (17-HGL-DTGs, Heiling et al. 2010), and caffeoylputrescine (CP, Kaur et al. 2010). Because we were interested in the effect of entrainment on the induced plant defense response, we show targeted metabolite data for the time point after 3 d herbivory ($n = 5$ plants, Figure 4). These defense metabolites accumulated to similar levels for in-

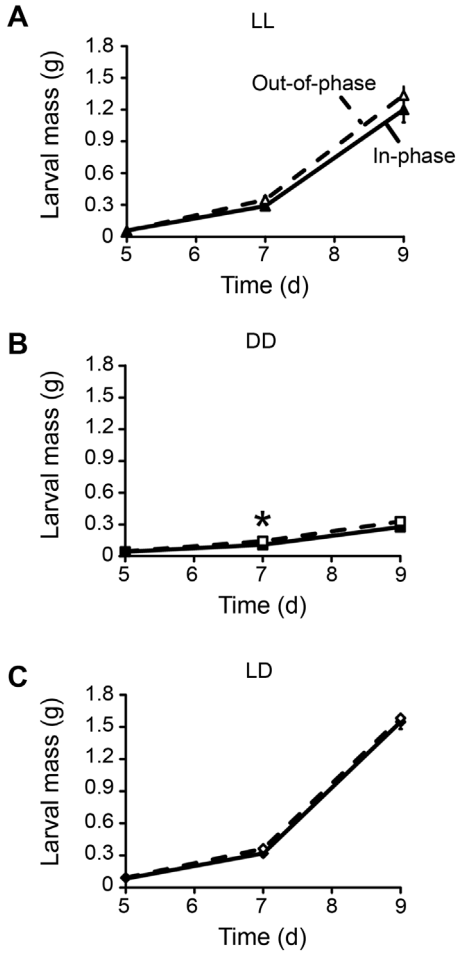

Figure 2. *Manduca sexta* larvae grow similarly on in-phase and out-of-phase *Nicotiana attenuata* plants under constant or diurnal conditions

M. sexta growth was monitored from day 5, to avoid increasing mortality of very young larvae, until day 9 (corresponding to the 2nd through 4th of 5 instars). (**A**) In LL, larval performance was similar on in-phase and out-of-phase plants for all three timepoints. (**B**) In DD larvae on out-of-phase plants were significantly heavier (*P-value < 0.05) on day 7, but not for the other two timepoints. Furthermore, final larvalmass on day 9 was much lower in DD than in LL or in LD. (**C**) In LD, larval performance was similar at all three timepoints and larval had a similar finalmass on day 9 as in LL. Thus, larval growth did not differ overall on in-phase versus out-of-phase treatments under any of the three light conditions. Wilcoxon rank-sumtests were performed to compare treatments within each time point and regime. Shown are means ±*SE*; for constant light (LL), n = 23–29; for constant dark (DD), n = 30–37; and for diurnal conditions (LD), n = 35–46 larvae. Treatment labels in panel (a) apply to all charts. Weighing of larvae coincided with the exchange of plants (see Figure 1).

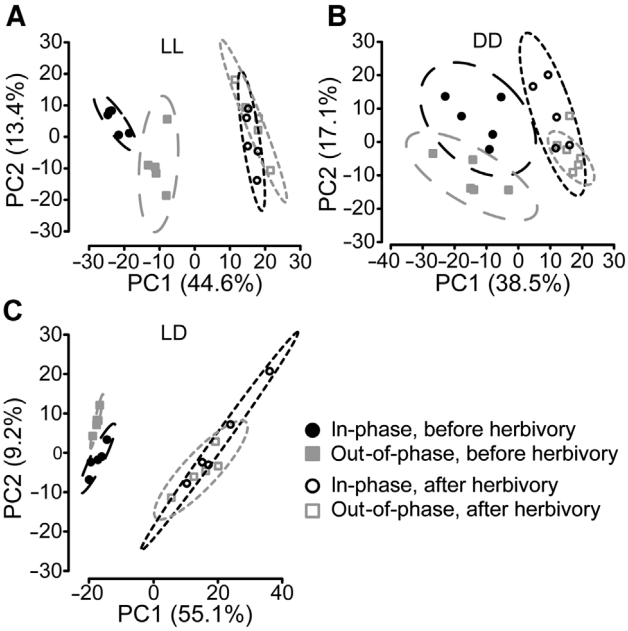

Figure 3. In-phase and out-of-phase *Nicotiana attenuata* plants respond similarly to *Manduca sexta* attack under constant or diurnal conditions

Principal component analyses (PCAs) were conducted on metabolomic fingerprints of plant samples (n = 5 plants/treatment) taken before or after *M. sexta* infestation for each regime. (**A**) In LL, confidence intervals of treatment groups (in-phase and out-of-phase) did not overlap before herbivory, but did so after herbivory. (**B**) However, in DD, confidence intervals overlapped before and after herbivory, although the zone of overlap grew after herbivory. (**C**) Similarly, in LD, confidence intervals overlapped after, but not before herbivory. Overall, in-phase and out-of-phase plants became similar after 3 d herbivory under the respective light regimes. Filled symbols stand for samples collected before herbivory, and empty symbols stand for samples collected after 3 d herbivory, under the respective light regime. Dashed and dotted lines delineate 95% confidence intervals; groups for which confidence intervals do not overlap are considered significantly different. Treatment groups differed due to induced defense in response to 3 d of herbivory (principal component 1, PC1) and effects due to prior entrainment conditions (PC2) as well as by the effects of continued exposure to the respective light regime (PC1 and PC2), and interactions of these factors (see also PCAs in supplementary information for sample groups collected before pre- infestation treatment; similar effects explain the separation along PC1 and PC2 in those cases).

phase and out-of-phase plants under LL and LD, except for CP under LL (Figure 4, t = –2.649, df = 8, $P = 0.0293$, all other P-values > 0.17, Table S2). Accumulation of CP was in fact lower for in-phase than out-of-phase LL plants, which is not consistent with the hypothesis that in-phase plants are better defended against herbivores.

However, in-phase plants accumulated significantly more nicotine and HGL-DTGs than out-of-phase plants under DD (Figure 4, nicotine: t = 2.901, df = 8, $P = 0.0199$; HGL-DTGs: t = 2.327, df = 8, $P = 0.0484$), consistent with the anticipatory defense hypothesis. In DD, levels of HGL-DTGs were very low in comparison to levels in plants grown under LL and LD conditions, and CP accumulation was too low to quantify. In

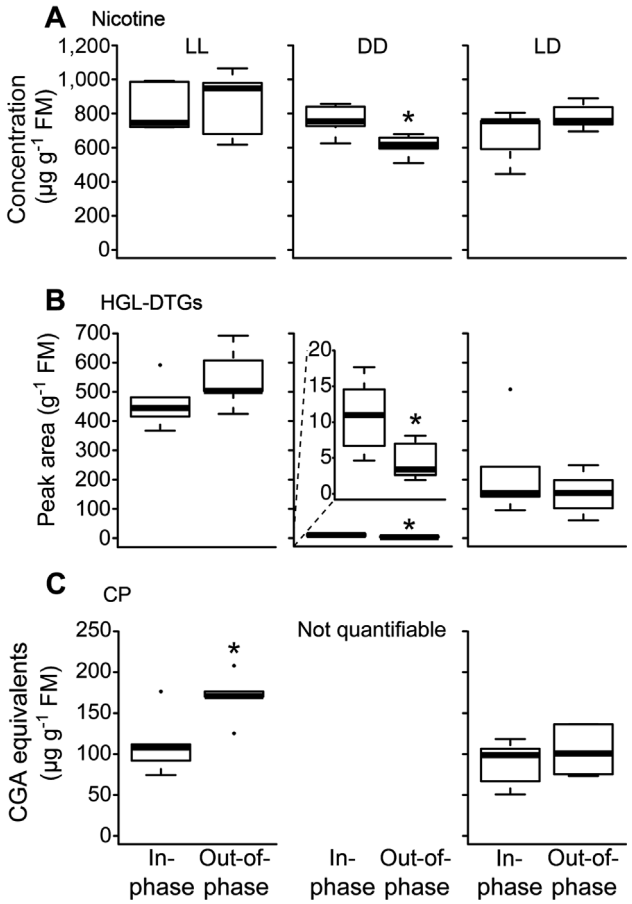

Figure 4. Specific herbivory-induced defense metabolites in leaves of *Nicotiana attenuata* respond differently to entrainment as well as light regime

Concentrations of nicotine (**A**), total hydroxygeranyllinalool diterpene glycosides (HGL-DTGs, **B**) and caffeoylputrescine (CP, **C**) in in-phase or out-of-phase plants after 3 d of *M. sexta* infestation ($n = 5$ plants/treatment). To facilitate comparisons between different light regimes, panels within a compound are shown with the same scale, but not for HGL-DTGs in DD (inset). Nicotine and HGL-DTGs differed significantly ($^*P < 0.05$) between in-phase and out-of-phase plants under DD while CP differed significantly under LL; all in-phase and out-of-phase pairs were compared using Student's *t*-tests, and no other significant differences were found. FM, fresh mass.

fact, irrespective of entrainment, HGL-DTGs decreased over time in plants under DD (mean ± SE, $n = 5$, in relative peak area/g FM, before placement in DD: in-phase 66.4 ± 8.3, out-of-phase 36.8 ± 6.3; after 24 h DD: in-phase 41.5 ± 9.1, out-of-phase 20.6 ± 4.9; after 3 d herbivory (4 d DD): in-phase 10.9 ± 2.4, out-of-phase 4.6 ± 1.2); in contrast, nicotine accumulation under DD was similar to levels under LL and LD. These subtle differences in metabolites between in-phase and out-of-phase plants under DD did not affect *M. sexta* growth (Figure 2, Table S1). Rather, they highlight how constant conditions might dampen or alter defense induction, as does the fact that plants under LL accumulated HGL-DTGs to higher levels than plants under LD (Figure 4).

Feeding patterns of *M. sexta* larvae under diurnal and constant conditions

Previous studies have indicated a strong dependence of *M. sexta* activity on temperature (Kingsolver and Woods 1997; Petersen et al. 2000). Lamps in our climate chambers emit infrared as well as visible light, and we hypothesized that the infrared irradiation especially might have increased the temperature of chambers during light periods when compared to dark periods. In the *M. sexta* growth assays, neonates initially placed on the adaxial surface of rosette leaves could be found all over the plant several days later, frequently sitting on the abaxial side or on the edge of leaves. Furthermore, observations of caterpillar behavior in the glasshouse, as well as in the field (e.g., Casey 1976), show that *M. sexta* larvae prefer to sit hidden under leaves. We thus measured temperatures below the surface of plants' leaves, at four different positions in each of the two climate chambers for at least 2 d, using pendant data loggers. Temperatures during dark periods reflected the set temperatures of the climate chambers (approximately 24 °C), but temperatures under leaves during light periods deviated from set temperatures by approximately 3 °C (Table S3).

We checked for diurnal fluctuation in *M. sexta* larval feeding activity over 24 h, following a 24 h acclimation period under DD, LL, and LD conditions and temperatures: LL at 27 °C, DD at 24 °C, and LD with a changing regime of 12 h L (26 °C):12 h D (24 °C). Larvae were fed on freshly cut *N. attenuata* rosette leaves from a common pool of plants under in-phase LD conditions. After 24 h of acclimation, leaf area removed by larvae over an additional 24 h in six 4 h periods was quantified as a measure of feeding activity ($n = 5$–6 larvae; Figures 5, S4).

Damage increased over the 24 h of the experiment under LL with a leveling-off toward the end of the experiment, which can likely be attributed to the initiation of molting by larvae (Figure 5A). Similarly, there was an increase in damage over the course of the experiment under DD without discernible fluctuations in inflicted damage (Figure 5B). Damage in leaf area removed increased with larval mass as larvae grew (Table S4), so the total amount of leaf area removed increased over 24 h, and the low slope of the increase in damage rates under the constant conditions highlights the strong interdependence of larval size and damage.

Under LD conditions, damage rates increased over the first three time intervals, but dropped in the 4th interval which followed the switch to the night regime, to increase again during the night (Figure 5C). However, in the 5th and 6th time intervals, in the middle and end of the night period, damage rates increased again. When normalized to larval mass, damage rates measured under LD conditions in the 1st time interval, from 9:00 to 13:00, and in the 4th, 5th and 6th time intervals, from 21:00 to 09:00,were significantly lower than rates measured in the 2nd and 3rd time intervals, between 13:00 and 21:00 (Figure S4, minimal linear mixed effects model, likelihood ratio: 26.36, $P < 0.0001$). In contrast, damage under LL and DD conditions occurred at a nearly constant rate per unit larval mass (Figure S4).

Thus, damage inflicted to leaves by *M. sexta* larvae rapidly and steadily increased with increasing caterpillar mass under all conditions, with evidence of diurnal fluctuation. Feeding damage reached the highest rates under LL and was lowest under DD conditions regardless of whether rates were

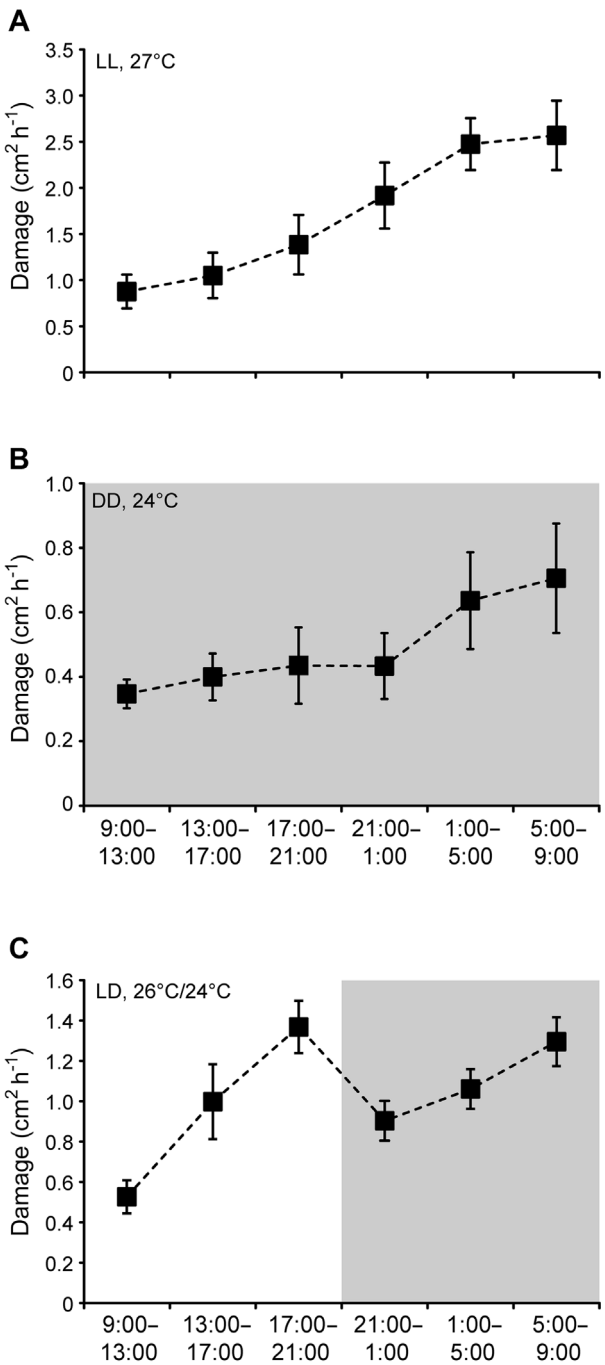

Figure 5. Damage by *Manduca sexta* larvae feeding on leaves of *Nicotiana attenuata* increases under all regimes
Damage to *N. attenuata* leaves caused by five 2nd-instar *M. sexta* larvae after 24 h acclimatization under the three experimental regimes used (see Figure 1), mean ± SE (n = 5–6). Note that Y-axes have different scales. Overall, damage from *M. sexta* larvae (consumed leaf area in cm²/h) increased two- to fourfold within 24 h under all three regimes (**A–C**). However under LD conditions, the amount of damage decreased initially after the onset of the dark phase (**C**). For choice of temperatures, see Results text and Table S3. Damage normalized to larval mass is shown in Figure S4; masses are given in Table S4.

corrected for larval mass (Figures 5, S4). This directly correlates with the differences in larval growth rates (Figure 2), changes to metabolic fingerprints under herbivory (Figure 3), and observed levels of HGL-DTGS and CP (Figure 4) under LD, LL, and DD.

DISCUSSION

Goodspeed and colleagues (Goodspeed et al. 2012, 2013a, 2013b) have proposed an "anticipatory defense" effect of enhanced plant resistance to insect herbivores conferred by correct entrainment with the environment and thus herbivore activity. This effect has so far been observed only with the generalist *Trichoplusia ni*, which displays circadian feeding activity on artificial diet (Goodspeed et al. 2012). Here, we show a counterexample, in which co-entrainment with the specialist herbivore *Manduca sexta* does not benefit the wild tobacco *Nicotiana attenuata*. Consistent with reports of physiological stresses experienced by plants under constant light or dark conditions (Graf et al. 2010; Sysoeva et al. 2010), our results also indicate that stress caused by constant experimental conditions may interfere with plant defense responses and thus confound results obtained under such artificial light and temperature regimes and thwart conclusions about the role of the clock in a plant's anti-herbivore defense.

N. attenuata plants induced defense against *M. sexta* regardless of plant and larval entrainment

Overall, *M. sexta* larval growth was similar on in-phase and out-of-phase *N. attenuata* plants under three different experimental set-ups (Figure 1) using constant dark (DD), constant light (LL), or diurnal (LD) conditions, except for a small and transient, but significant increase in mass of larvae feeding on out-of-phase plants under DD. Larval growth was, however, slower under DD conditions, perhaps due to reduced feeding activity (Figures 5, S4) or less nutritious plant material (Figure S3). Lower temperatures under DD, in the absence of warming infrared radiation from lamps, might have reduced larval growth rates (Figure 5, Table S3; Kingsolver and Woods 1997; Petersen et al. 2000). Metabolomic fingerprints of *N. attenuata* plants changed markedly after 3 d of feeding by *M. sexta* larvae under all three conditions, and there was little difference between in-phase and out-of-phase plants after herbivory, even though these groups differed prior to herbivory under LL and LD conditions (Figure 3). However, metabolomic fingerprints from plants in DD were generally less affected by herbivory than were those from plants in LL or LD (Figure 3). Accumulation patterns of HGL-DTGs and CP in particular (Figure 4) suggest that these compounds depend strongly on light and, likely, available carbon (Figure S3; Wünsche et al. 2011).

M. sexta feeding activity increases rapidly over time

M. sexta larvae usually remain on plants from oviposition until they reach at least the third of five to six larval instars (e.g., Kingsolver and Nagle 2007). The green larvae are mobile within their host plants, using them as camouflage, adopting postures similar to branches and hiding beneath leaves, which might also help them to thermoregulate in their native desert

habitat (as shown for *M. quinquemaculata*; Kessler and Baldwin 2002b); and indeed the timing of feeding bouts in nature may be largely independent of temperature (Casey 1976; Bernays and Woods 2000) even though the rate of growth and development is not (Kingsolver and Woods 1997; Petersen et al. 2000; Kingsolver and Nagle 2007). The larvae, if they survive, may not leave the plant until there is little plant material left (Schittko et al. 2000).

Perhaps *N. attenuata* plants benefit most from inducing defense to the maximum possible level at maximum speed in response to *M. sexta* attack, anticipating the rapid growth of sedentary larvae rather than diurnal fluctuations in their activity. In this study, under all conditions, *M. sexta* damages rates increased by two- to five-fold within 24 h as larvae grew (Figure S4). This rapid increase in damage with increasing size of larvae might mask temperature- and daytime-specific fluctuations; for example, feeding rates of second-instar *M. sexta* under LD conditions transiently decreased at the beginning of the dark phase, and this pattern was more apparent when damage rates were normalized to increasing larval size (Figures 5, S4). This decrease was likely due to diurnal temperature fluctuations; when *M. sexta* larvae were exposed to a 12 h:12 h LD regime at constant temperature, there was a fairly constant proportion of active animals over 24 h (Casey 1976). Differences in the magnitude of damage rates between the three settings (i.e., leaf area removed was greatest in the LL setting and was lowest in the DD setting; note different scales in Figure 5) are probably due to a combination of slightly different larval growth rates, and different incubation temperature, because increased temperatures elevate feeding rates and, consequently, increase developmental rates of *M. sexta* larvae (Kingsolver and Woods 1997; Petersen et al. 2000).

Typical temperature fluctuations in *N. attenuata*'s native environment of the Great Basin Desert in UT are much stronger than those in our climate chambers: in spring, when *M. sexta* eggs are laid on plants, temperatures may fluctuate by more than 20 °C over 24 h. It will be interesting to investigate whether this large temperature change results in predictable diurnal fluctuations in *M. sexta*'s feeding behavior in nature. If so, plants with a functioning circadian clock might in fact be able to anticipate diurnal rhythms of *M. sexta* feeding activity. The question is whether they would benefit from doing so, given *M. sexta*'s rapid growth and the likelihood that plants in nature are attacked by a varied community of other herbivores (see for example Schittko et al. 2000; Glawe et al. 2003; Steppuhn et al. 2004; Steppuhn and Baldwin 2008; Jander 2012).

When do plants anticipate attack, and when do they react to attackers?

In contrast to pathogen attack, which is strongly correlated to diurnal humidity and temperature cycles (Bhardwaj et al. 2011, Wang et al. 2011), herbivore activity may be less predictable on a daily time scale within a given environment. Rather, it is an intra- and interspecifically plastic trait subject to modification by external factors such as predator activity and availability of mating partners, and internal factors like nutritional status and hormones, in addition to the common circadian zeitgebers of light and temperature (Wigglesworth 1972; Jander 2012). In nature, a single plant is often infested by multiple herbivore species (Denno et al. 1995). Variation in climate and other environmental factors among habitats, and previous attack by particular herbivores, may be more reliable predictors of future herbivore attack than are plastic activity peaks, resulting in genetic and epigenetic adaptation of plant populations (Agrawal et al. 2012; Rasmann et al. 2012).

Nevertheless, some plant–herbivore pairs may be affected by circadian fluctuations in herbivore activity and plant defense capacity: *Arabidopsis thaliana* plants were more resistant to *T. ni* herbivores under both DD and LL conditions when plants and herbivores had been co-entrained (Goodspeed et al. 2012). In the case of *A. thaliana* and *T. ni*, it is difficult to interpret this interaction in an evolutionary context, since *T. ni* is a broad generalist, these two organisms have not co-evolved, and thus circadian fluctuations in *A. thaliana*'s defense response cannot be said to occur specifically in anticipation of *T. ni* feeding (Jander 2012).

One way to reconcile our data with those from Goodspeed et al. (2012, 2013b) is to hypothesize that these studies collectively describe a previously unrecognized difference between constitutive or generally damage-induced, and specifically herbivore-induced plant defenses (Bonaventure 2014). Constitutive production of plant defense metabolites may be, at least in part, regulated by the circadian clock (Kerwin et al. 2011); and in the absence of reliable, specific, contraindicative information about attackers, plants may maintain these endogenous rhythms, as when the generalist *T. ni* attacks *A. thaliana* or harvested fruits and vegetables (Goodspeed et al. 2012, 2013b). However, if plants receive specific and reliable information about the identity of an attacker, they may be likely to engage in a targeted response which overrides constitutive rhythms.

A similar phenomenon has already been demonstrated for plant immunity: Wang et al. (2011) showed that *A. thaliana* plants rhythmically pulse the transcription of genes involved in basal and R-gene-mediated defense under the control of a core clock gene, the MYB transcription factor *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*); but following attack by the oomycete *Hyaloperonospora arabidopsidis*, the R-gene-mediated response disrupted the rhythmicity and increased the amplitude of *CCA1* gene expression, thus inducing sustained immune gene expression. The mechanisms behind the involvement of the clock in immunity have been further investigated by a handful of research groups using different strains of *H. arabidopsidis*, *P. syringae*, and pathogen-associated molecular patterns (PAMPs) (Bhardwaj et al. 2011; Wang et al. 2011; Zhang et al. 2013). A picture is emerging in which components of basal and R-gene-mediated defense fluctuate with individual circadian phases (expression of defense-related genes versus stomatal opening cycles), while specifically elicited responses may feed back on the clock to abolish constitutive rhythmicity – as has been shown for glucosinolate production in *A. thaliana* (Kerwin et al. 2011). This can be interpreted as an articulation of the long-known difference between constitutive and induced plant defenses, informed by an understanding of plant circadian clocks and their role in coordinating plant's activities (Kessler and Baldwin 2002a; Harmer 2009; Kerwin et al. 2011; Wang et al. 2011).

Interestingly, jasmonic acid (JA) and salicylic acid (SA) levels fluctuated constitutively in *A. thaliana* plants under DD

and LL, in oppositely phased circadian cycles. Herbivory by *T. ni* increased the total amount of JA, but did not alter this rhythm, in contrast to the observations of altered rhythm following induction in plant–pathogen interactions (Wang et al. 2011; Zhang et al. 2013). The JA peak in wild-type (WT) plants preceded a maximum of feeding activity of *T. ni* towards the end of the subjective day and thus could have indicated anticipation of attack (Goodspeed et al. 2012). In accordance with this pattern of JA fluctuation, Shin et al. (2012) showed in *A. thaliana* that MYC2, a transcription factor which mediates jasmonate signaling, oscillates in transcript levels as well as protein levels throughout the day with a maximum toward dusk.

An alternative hypothesis is that the constitutive, circadian-controlled opposed oscillation of jasmonates and salicylates identified by Goodspeed et al. (2012, 2013a) is a general defense priming mechanism in *A. thaliana*, but not in *N. attenuata*. Many snail and slug species are known to attack *A. thaliana*, and these ravenous herbivores are both highly mobile, and generally nocturnal (Falk et al. 2013). Application of the locomotion mucus of at least one snail species to wounded *A. thaliana* leaves is known to induce accumulation of jasmonates (Falk et al. 2013), but many slugs and snails also induce salicylate-related pathways (Meldau et al. 2014), presumably via the pathogens that reside in their mucus. Attack by molluscs at night might mean a greater risk of infection with pathogens. As for attack by pathogens, attack by molluscan herbivores is also highly dependent on predictable diurnal fluctuations in temperature and humidity, and the circadian clock is implicated in balancing pathogen defense with growth in *A. thaliana* (Zhang et al. 2013). Perhaps *A. thaliana* has evolved to anticipate more regular and predictable patterns of attack than the opportunistic, ephemeral desert annual *N. attenuata*.

CONCLUSIONS

Nicotiana attenuata supports a diverse herbivore community, among which the most damaging insect herbivore, *Manduca sexta* may have an activity pattern dominated by rapidly increasing, rather than diurnally predictable activity. While defense responses to herbivores can reduce growth in *N. attenuata* (reviewed in Steppuhn and Baldwin 2008), plants that induce defense against herbivores also are fitter than those that do not (e.g., Zavala and Baldwin 2004), and thus evolutionary pressure may push *N. attenuata* plants not to anticipate attack, but to rapidly respond when it occurs. *N. attenuata* relies on constitutive as well as induced defense: for example, nicotine and 17-HGL-DTGs have both a constitutive and induced component (Baldwin 1999; Heiling et al. 2010); but the induced component dominates in magnitude and complexity (Wu and Baldwin 2010; Gaquerel et al. 2010), suggesting that constitutive fluctuations in *N. attenuata*'s defenses may be obviated by a strong induced response, which might be less affected by the plant's internal rhythm. To the extent that there are diurnal fluctuations in *N. attenuata*'s induced defense metabolites (Kim et al. 2011), these may be indicative of physiological coordination between general and specialized metabolism and might have little effect on plant resistance to herbivores. Studies using transgenic *N. attenuata* plants with altered circadian clock components may allow a closer investigation of the relationship between plant and insect diurnal rhythms, in the natural conditions under which *N. attenuata* evolved.

MATERIALS AND METHODS

Materials and methods for supplementary data, and expanded explanations of experimental and analysis procedures, are given in a supplementary materials and methods section.

Cultivation of *Nicotiana attenuata* plants

All experiments were performed with *N. attenuata* Torr. Ex. Wats. (Solanaceae) wild-type (WT) plants from a 31st-generation inbred line originating from a natural population at the Desert Inn Ranch in Utah, UT, USA in 1988 (Glawe et al. 2003). Germination and growth conditions until potting were as previously described (Krügel et al. 2002; Schuman et al. 2014). Twenty days after germination, individual small rosette plants were transplanted to 1 L plastic pots containing FrühsdorferNullerde (HawitaGruppe, Vechta, Germany) with 0.5 g/LPG mix (Yara UK Limited, N E Lincolnshire, UK), 0.9 g/L $Ca(H_2PO_4)_2$, 0.35 g/L $MgSO_4 \cdot 7\,H_2O$ (Sigma–Aldrich Chemie GmbH, Munich, Germany), and 0.055 g/L Micro Max (Everris GmbH, Nordhorn, Germany), and cultivated in one of two climate chambers (Johnson Controls Unitary Products, Norman, OK, USA; dimensions: 4.00 m l x 2.22 m w x 2.35 m h) under either an in-phase regime (light 09:00–21:00/dark 21:00–09:00) or an out-of-phase regime (dark 09:00–21:00/light 21:00-09:00) at 65% RH and 24 °C for at least another 10 d prior to the start of experiments. The 12 h light period reflects a typical photophase for *N. attenuata* plants during vegetative growth and flowering (May–June), based on PAR measurements taken in the wash in Southwestern UT in which we conduct field research, which is part of *N. attenuata*'s native range (Y. Joo and colleagues, in preparation). Preliminary experiments demonstrated that *N. attenuata* plants flower under both long-day (16 h light/8 h dark) and 12 h light/12 h dark conditions. Light in climate chambers was provided by Master Sun-T PIA Agro 400 W (Koninklijke Philips N.V., Eindhoven, The Netherlands) at 100% intensity, approximately 400 μmol/m^2 per s PAR (measured using a LI-COR LI-250 A Light Meter und Sensor LI-COR Quantum Q 35195, LI-COR, Lincoln, NB, USA). Plants were watered initially with 200 mL per plant, and from then on daily with 100 mL per plant. Peter's Professional Allrounder 20-20-20 N-P-K + TE fertilizer (Everris) was added once per week at a concentration of 0.75 g/L. Plants were used for the larval growth experiments under constant dark (DD), constant light (LL), or in-phase diurnal (LD) conditions after entrainment. To quantify larval feeding activity, leaves were cut every 4 h from a designated batch of rosette-stage plants cultivated under in-phase LD conditions, and batches were not re-used.

Manduca sexta larvae

Manduca sexta eggs originated from an in-house colony at the Max Planck Institute for Chemical Ecology in Jena. Larvae hatched within 5 d after oviposition. For growth assays, eggs

were incubated under the in-phase entrainment conditions in the climate chamber for at least 2 d, and for an additional 1 d under either LL or DD for experiments under constant conditions (Figure 1). Egg collection and incubation were timed such that most eggs hatched within the last 12 h of incubation. At the end of incubation, featherweight forceps were used to place freshly hatched *M. sexta* neonates on leaves.

M. sexta growth assays

We decided not only to emulate the experimental set-up of Goodspeed et al. (2012) under constant conditions but, additionally, we established a set-up under diurnal conditions in which plants entrained under the out-of-phase regime (12 h:12 h dark: light, DL) were transferred to the in-phase regime (12 h:12 h LD) and kept under in-phase conditions for 2–3 d. A detailed justification is given in the supplementary materials and methods. Plants were entrained under either in-phase or out-of-phase conditions (see cultivation of *Nicotiana attenuata* plants) and *M. sexta* eggs were entrained under in-phase conditions (see *Manduca sexta* larvae). When *N. attenuata* plants enter the flowering stage, herbivory-induced defense responses are reduced (Diezel et al. 2011), but small rosettes would not have provided sufficient food for *M. sexta* larvae. We therefore used plants that were in full rosette to early elongating stages (31–33 d post-germination) at the start of assays. Larvae were kept under LL, DD, or in-phase LD conditions after entrainment until the end of the assay and transferred to new, entrained plants every 2–3 d to reduce habituation of plants to experimental conditions, or reduce the exacerbation of off-target effects due to prolonged exposure to constant conditions (Graf et al. 2010; Sysoeva et al. 2010; Jänkänpää et al. 2012). All plants were from the same synchronously germinated batch, so plants and larvae both aged over the course of the assay.

For diurnal (LD) conditions, at 9:00 on the first day of the experiment (lights-on in the in-phase chamber, lights-off in the out-of-phase chamber), 10 plants were transferred from the out-of-phase to the in-phase chamber. Six recently hatched *M. sexta* larvae were placed per plant on 10 in-phase and 10 out-of-phase *N. attenuata* plants (see Figure 1); larvae were distributed evenly among three similar, fully-developed rosette leaves on each plant. Plants of the same treatment were kept together in trays (five plants/tray) distributed in the chamber, so that if larvae moved between plants, they were most likely to move onto a plant of the same treatment. After 3 d, 20 new out-of-phase plants were transferred to the in-phase chamber and plants in each treatment were exchanged for undamaged plants with the respective prior entrainment. Two to three larvae (if possible, from the same plant) were placed together on a new plant of the same treatment. Due to mortality, some larvae were lost and from day 4 some also moved between plants, and thus larvae, not plants, were treated as biological replicates. Subsequent exchanges of plants were conducted every 2 d as described. At the second exchange of plants (5 d) and at all remaining exchanges (7 and 9 d), larvae were weighed, since younger larvae experienced higher mortality after weighing; mass was determined to $\pm$ 0.001 g with a laboratory balance (Sartorius AG, Göttingen, Germany). Larval growth is interpreted as an indicator of both larval performance and plant resistance (e.g., Zavala and Baldwin 2004). The experiment was terminated when the largest larvae (4^{th} or early 5^{th} instar) began actively looking for host plants outside the trays containing plants of a single treatment.

Constant conditions were achieved using two climate chambers set to opposite 12 h:12 h LD cycles (the same chambers used simultaneously for in- and out-of-phase entrainment), so that plants for exchange remained entrained to the correct light regime, while organisms could be exposed to DD or LL by transferring plants and larvae between chambers every 12 h. For pre-infestation treatment and *M. sexta* infestation in DD, transfer without exposure to light was achieved by placing organisms in lightproof containers at the end of the 12 h dark phase and then transferring them to the other climate chamber after it entered the dark phase. Three green LED lights ($\lambda = 520$ nm, each 1 W) were used for illumination of entire chambers during experimental measurements under DD, and curtains were hung in front of chamber doors to avoid accidental light exposure from outside. For LL, organisms were transferred uncovered between chambers every 12 h. Experimental setups under DD and LL were conducted simultaneously. One day before *M. sexta* infestation, at 9:00 (lights-on in the in-phase and lights-off in the out-of-phase chamber), five each of in-phase and out-of-phase plants were transferred from their prior entrainment regime to the respective constant conditions. This 1 d pre-infestation exposure to constant conditions was done to avoid differences due to confounding effects from prior entrainment regimes (e.g., nutritive state; Goodspeed et al. 2012). Simultaneously, *M. sexta* eggs were also transferred to constant conditions. After 1 d under constant conditions, freshly hatched *M. sexta* larvae were placed on three fully developed rosette leaves of *N. attenuata* WT plants: nine neonates/plant on five plants/treatment in each chamber; larvae were placed as described for diurnal conditions. After 3 d, plants in each treatment were exchanged for undamaged plants with the same entrainment and 1 d pre-infestation treatment in constant conditions. At the first exchange of plants (3 d), 10 plants of each treatment were used, so densities were reduced to four to five larvae/plant (if possible larvae were transferred from the same previous plant). In subsequent exchanges of plants, we tried to even out the number of larvae per plant as number of larvae decreased due to mortality, and three to four from one plant were placed together on a new plant. At the second exchange of plants and at all remaining exchanges, the larvae were weighed as described for LD. Again, the first exchange occurred 3 d after infestation while subsequent exchanges occurred at 2 d intervals (days 5, 7 and 9).

Plant tissue samples from *M. sexta* growth assays

For LD, one fully developed rosette leaf was collected from each plant before infestation with *M. sexta*, and after 3 d of herbivory, either the most heavily attacked, fully developed rosette leaf was collected, or a similar amount of leaf tissue was pooled from multiple such leaves, if these leaves all had similar amounts of tissue removed by *M. sexta*. For DD and LL, leaves at position 0 (oldest leaf undergoing the transition between sink and source) were collected before the 1 d pre-infestation treatment in constant conditions; leaves at position +1 (youngest source leaf) were collected prior to

infestation with *M. sexta*, and leaves were collected as described for LD after 3 d of *M. sexta* infestation. The same plants were thus sampled two to three times. Midveins were removed, because small larvae normally do not feed on these leaf parts, and leaves were flash-frozen in liquid nitrogen and stored at −80 °C. Tissue was ground and aliquoted over liquid nitrogen immediately before extraction. Plants were treated as biological replicates for measures of plant metabolites.

Extraction of plant metabolites for fingerprinting and targeted analysis

Per tissue sample ($n = 5$ plants), 55 ± 5 mg of frozen, ground leaf material were weighed into 2 mL Eppendorf tubes and masses were recorded (±0.0001 g weighed with a balance from Sartorius AG). Extraction of metabolites was performed as described by Gaquerel and colleagues (2010) using 500 µL extraction buffer without internal standards (40% methanol (HPLC grade; VWR International Ltd., Leicestershire, UK), v/v, containing 40 mM acetic acid (MerckKGaA, Darmstadt, Germany) and 44 mM ammonium acetate (Sigma–Aldrich); pH 4.8). Supernatants were transferred to a 1.5 mL HPLC crim*P* vial N11-1 H*P* with a 250 µL micro-insert (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Metabolomic fingerprinting of plant tissue extracts

Plant extracts were analyzed by liquid chromatography-electrospray ionization-micro time of flight-mass spectrometry (LC-ESI-microToF-MS, Bruker Daltonik GmbH, Bremen, Germany) in positive mode as described by Gaquerel and colleagues (2010), but with specific modifications of the procedure as detailed by Kim and colleagues (2011) and with a 2 µL injection volume. Raw data files from the Bruker software (Data Analysis v4.0) were exported as net CDF format and were processed as described by Kim and colleagues (2011), including peak detection, retention time correction, and annotation of isotope and adduct ions using the bioconductor XCMS and CAMERA packages (http://www.bioconductor.org/; Smith et al. 2006; Tautenhahn et al. 2008; Benton et al. 2010). XCMS output files were processed using Microsoft Excel 2010. We deleted all m/z features with a retention time ≤40 s (calibration fluid), as well as features within the last 30 s of the chromatogram (contaminants). We selected single abundant features for each compound by removing ions identified as low-abundance isotopes ((M + 1), (M + 2), etc.). Peaks were set to 0 if they had a relative intensity ≤90 or if their specific compound did not appear in 3/4 of the replicates of one treatment. Peak intensities of samples were normalized to g fresh mass. After processing, the data sets comprised 693 m/z features for LL, 604 m/z features for DD, and 654 m/z features for LD.

Quantification of target plant specialized metabolites

Plant extracts were also analyzed on a high-pressure liquid chromatograph coupled to a UV diode array detector (HPLC-DAD, Agilent Technologies, Santa Clara, CA, USA) as described by Oh et al. (2012). Peak areas were integrated using Chromeleon (version 6.8; Dionex Corporation, Sunnyvale, CA, USA) and the amount of metabolites in plant tissue was calculated by comparison with external standard curves of nicotine, chlorogenic acid (CGA), and rutin (Sigma–Aldrich; 7.8, 15.6, 31.3, 62.5, 125 or 250 µg/mL of each standard). To account for fluctuating measurement conditions, external standard curves were compared before, between, and after samples. Concentration values below the range of the standard curves were expressed as not quantifiable. Caffeoylputrescine (CP) was estimated based on external CGA calibration and expressed as µg CGA equivalents. HGL-DTG content was expressed as relative peak area. The amount of metabolites in the plant tissue was expressed as amount per g fresh mass (FM).

Quantification of feeding activity

We quantified feeding activity as $cm^2 \cdot g$ larval mass/h under the same light and temperature conditions used for *M. sexta* growth assays. Damage and larval mass were measured in 4 h intervals over 24 h, immediately following a 24 h acclimation period. We employed temperature/light data loggers to determine accurate temperatures experienced by *M. sexta* larvae during growth assays (HOBO UA-002-64 Pendant Temp/Light, Onset Computer Corporation, Cape Cod, MA, USA, accuracy: temperature: ± 0.53 °C from 0 °C to 50 °C; logging interval: 10 min; logging time: at least 2 d). Because *M. sexta* larvae are often found under leaves, we placed the loggers shaded under rosette leaves on soil of potted plants. Loggers were placed in four positions distributed throughout each of the two climate chambers used for *M. sexta* growth assays.

We used Vötsch growth chambers (VB 1014, Vötsch Industrietechnik GmbH, Balingen-Frommern, Germany) with the following settings, determined by temperatures measured as described above: for LL, 24 h light at 27 °C; for DD, 24 h dark at 24 °C; and for LD, 12 h light at 26 °C/12 h dark at 24 °C; humidity was set to 65% as in the climate chambers used for growth assays. Precautions to avoid light exposure for DD conditions and the dark phase of LD conditions were similar to the *M. sexta* growth assays: chambers were shielded from external light sources with curtains across the doors; in the dark, during work in the growth chamber, red light at a wavelength thought to be invisible to *M. sexta* larvae was used ($\lambda = 720$ nm; Briscoe and Chittka 2001) and for the collection of rosette leaves in the dark phase of the climate chamber, green LED lights were used as previously described. Experiments began at 9:00, the time used to switch between light and dark periods in growth assays. *M. sexta* eggs were incubated for 3 d in an in-phase climate chamber (see *M. sexta* growth assays) and after hatching, 9–12 larvae/plant were placed on 5 plants and reared for 3–4 d until they reached the 2nd instar. The time was chosen such that caterpillars would not molt during the 24 h of quantification. Five larvae were placed in a square Petri dish with plant material ($n = 8$ dishes) and acclimated under the light and temperature conditions of the respective assay for 24 h. Leaf material was provided as food during acclimation and exchanged every 12 h; the bases of leaves were covered with water-soaked tissues to prevent water loss. Immediately following the 24 h acclimation, the larvae in each Petri dish were weighed together (see Table S4) and provided with two fresh rosette leaves. After each 4 h interval, larvae were again weighed, old leaves were replaced with two freshly cut rosette leaves as described, and old leaves were scanned to quantify consumed leaf area. A 1 cm^2 piece of millimetric paper was scanned alongside leaves as a size standard. Lines were drawn with the line tool in Adobe

Photoshop (version CS2, Adobe Systems GmbH, Munich, Germany) if necessary to re-construct missing portions of the edges of leaves, and the wand tool was used if necessary to remove shadows originating from scanning; then missing leaf area was quantified with SigmaScan (SigmaScan Pro – Image Analysis, Version 5.0.0 Copyright 1987–1999 SPSS Inc., IBM, Armonk, NY, USA; in "fill" mode, with the following settings – fill: threshold method: auto threshold: % range: 25). Leaf area removed was normalized by dividing by the actual time between exchanges of leaves (3.7–4.3 h), and dividing by the mass of larvae in the Petri dish at the start of the respective time interval. Of the 40 larvae in each condition, only one died under LL, three under DD, and two under LD conditions (mortality: 2.5%–7.5%) and the corresponding replicates were excluded from data analysis, as were replicates in which larvae did not begin to feed within the first 4 h of the experiment, or in which the majority of larvae began to molt; thus, the final number of replicates per assay was $n = 5–6$.

Statistical analyses

Statistical tests of differences in *M. sexta* growth, targeted defense metabolites, and *M. sexta* feeding damage rates were performed in R (version 2.12.2, 2.15.0, or 3.0.2) using RStudio (version 0.97.551) (R Core Team, 2012). Metabolomic fingerprinting data were analyzed in MetaboAnalyst (http://www.metaboanalyst.ca/, Xia et al. 2009, 2012, 2015): after first autoscaling to achieve normality and homoscedasticity, a principal component analysis (PCA) was conducted for all sample groups within a light regime (before transfer to constant conditions, before herbivory, and after 3 d herbivory) and, separately, only for the sample groups collected before and after 3 d herbivory within a light regime. Additionally, volcano plots were generated to filter for significant differences in the abundance of individual m/z features between leaf tissues of in-phase and out-of-phase plants after 3 d herbivory. Significant differences within an m/z feature were defined as a more than twofold change and a P-value < 0.05.

For *M. sexta* growth and targeted metabolite data, we checked treatment groups graphically (quantile-quantile plots) and statistically (Shapiro–Wilk tests) for normality, and used F tests to check for homoscedasticity. When the requirements of normality and homoscedasticity were met, Student's t-tests were used to test for differences between in-phase and out-of-phase plants. When data failed to meet one requirement and could not be made to meet these requirements by log transformation, we conducted Wilcoxon rank-sum tests for independent samples.

In order to investigate whether the leaf area consumed by *M. sexta* larvae differed between 4 h time intervals, and thus probably also light and dark periods, linear mixed effects models (lme; nlme package, Pinheiro et al. 2013) were performed. We corrected for the fact that heavier larvae consume more plant material using the leaf area removed per g initial larval mass per h as response variable (Table S4). Explanatory variables were the respective 4 h time intervals (fixed effect), and the replicate ID was set as a random intercept. The optimal fixed structure was found by stepwise model simplification and factor level reduction followed by the comparison of the models using likelihood ratio tests. This test was also used to obtain P-values. The minimal model was refitted with the restricted maximum likelihood method (REML) and validated for homogeneity of variances and normality of residuals (Zuur et al. 2009).

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AUTHOR CONTRIBUTIONS

J.H., S.M., S.K., Y.J., I.T.B., and M.C.S. designed experiments. J.H., S.M., Y.J., and M.C.S. conducted experiments. J.H. extracted and analyzed samples and J.H. and G.K. analyzed data. J.H. and M.C.S. wrote the first draft of the manuscript and all authors contributed to the revision and finalization of the manuscript.

REFERENCES

Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen J-P (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. **Science** 338: 113–116

Baldwin IT (1999) Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. **J Chem Ecol** 25: 3–30

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ (2005). Circadian rhythms from multiple oscillators: Lessons from diverse organisms. **Nat Rev Genet** 4: 121–130

Benton HP, Want EJ, Ebbels TMD (2010) Correction of mass calibration gaps in liquid chromatography-mass spectrometry metabolomics data. **Bioinformatics** 26: 2488

Bernays E, Woods H (2000) Foraging in nature by larvae of *Manduca sexta*-influenced by an endogenous oscillation. **J Insect Physiol** 46: 825–836

Bhardwaj V, Meier S, Petersen LN, Ingle RA, Roden LC (2011) Defence responses of *Arabidopsis thaliana* to infection by *Pseudomonas syringae* are regulated by the circadian clock. **PLoS ONE** 6: e26968

Bonaventure G (2014) Plants recognize herbivorous insects by complex signalling networks. In: Voelckel C and Jander G, eds. *Annual Plant Reviews Volume 47: Insect–Plant Interactions*. First edn. John Wiley & Sons, Ltd, West Sussex, UK. pp. 1–35

Briscoe AD, Chittka L (2001) The evolution of color vision in insects. **Ann Rev Entomol** 46: 471–510

Casey TM (1976) Activity patterns, body temperature and thermal ecology in two desert caterpillars (Lepidoptera: Sphingidae). **Ecology** 57: 485–497

Denno R, McClure M, Ott J (1995) Interspecific interactions in phytophagous insects: Competition reexamined and resurrected. **Ann Rev Entomol** 40: 297–331

Diezel C, Allmann S, Baldwin IT (2011) Mechanisms of optimal defense patterns in *Nicotiana attenuata*: Flowering attenuates herbivory-elicited ethylene and jasmonate signaling. **J Integr Plant Biol** 53: 971–983

Falk KL, Kästner J, Bodenhausen N, Schramm K, Paetz C, Vassão DG, Reichelt M, von Knorre D, Bergelson J, Erb M, Gershenzon J, Meldau S (2013) The role of glucosinolates and the jasmonic acid pathway in resistance of *Arabidopsis thaliana* against molluscan herbivores. **Mol Ecol** 23: 1188–1203

Gaquerel E, Heiling S, Schoettner M, Zurek G, Baldwin IT (2010) Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. **J Agr Food Chem** 58: 9418–9427

Glawe G, Zavala JA, Kessler A, Van Dam NM, Baldwin IT (2003) Ecological costs and benefits correlated with trypsin protease inhibitor production in *Nicotiana attenuata*. **Ecology** 84: 79–90

Goodspeed D, Chehab EW, Covington MF, Braam J (2013a) Circadian control of jasmonates and salicylates: The clock role in plant defense. **Plant Signal Behav** 8: 8–10

Goodspeed D, Chehab EW, Min-Venditti A, Braam J, Covington MF (2012) *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior. **Proc Natl Acad Sci USA** 109: 4674–4677

Goodspeed D, Liu JD, Chehab EW, Sheng Z, Francisco M, Kliebenstein DJ, Braam J (2013b) Postharvest circadian entrainment enhances cro*P* pest resistance and phytochemical cycling. **Curr Biol** 23: 1235–1241

Graf A, Schlereth A, Stitt M, Smith AM (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. **Proc Natl Acad Sci USA** 107: 9458–9463

Harmer SL (2009) The circadian system in higher plants. **Ann Rev Plant Biol** 60: 357–377

Heiling S, Schuman MC, Schoettner M, Mukerjee P, Berger B, Schneider B, Jassbi AR, Baldwin IT (2010) Jasmonate and ppHsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. **Plant Cell** 22: 273–292

Jander G (2012) Timely plant defenses protect against caterpillar herbivory. **Proc Natl Acad Sci USA** 109: 4343–4344

Jänkänpää HJ, Mishra Y, Schröder WP, Jansson S (2012) Metabolic profiling reveals metabolic shifts in *Arabidopsis* plants grown under different light conditions. **Plant Cell Env** 35: 1824–1836

Kallenbach M, Bonaventure G, Gilardoni PA, Wissgott A, Baldwin IT (2012) PNAS Plus: *Empoasca* leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. **Proc Natl Acad Sci USA** 109: E1548–E1557

Kaur H, Heinzel N, Schöttner M, Baldwin IT, Gális I (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. **Plant Physiol** 152: 1731–1747

Kerwin RE, Jimenez-Gomez JM, Fulo PD, Harmer SL, Maloof JN, Kliebenstein DJ (2011) Network quantitative trait loci mapping of circadian clock outputs identifies metabolic pathway-to-clock linkages in *Arabidopsis*. **Plant Cell** 23: 471–485

Kessler A, Baldwin IT (2002a) Plant responses to insect herbivory: The emerging molecular analysis. **Ann Rev Plant Biol** 53: 299–328

Kessler A, Baldwin IT (2002b) Manduca quinquemaculata's optimization of intra-plant oviposition to predation, food quality, and thermal constraints. **Ecology** 83: 2346–2354

Kessler D (2012) Context dependency of nectar reward-guided oviposition. **Entomol Exp Appl** 144: 112–122

Kim S-G, Yon F, Gaquerel E, Gulati J, Baldwin IT (2011) Tissue specific diurnal rhythms of metabolites and their regulation during herbivore attack in a native tobacco, *Nicotiana attenuata*. **PLoS ONE** 6: e26214

Kingsolver JG, Nagle A (2007) Evolutionary divergence in thermal sensitivity and diapause of field and laboratory populations of *Manduca sexta*. **Physiol Biochem Zool** 80: 473–479

Kingsolver JG, Woods HA (1997) Thermal sensitivity of growth and feeding in *Manduca sexta* caterpillars. **Physiol Zool** 70: 631–638

Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. **Chemoecology** 12: 177–183

Machado RAR, Ferrieri AP, Robert CAM, Glauser G, Kallenbach M, Baldwin IT, Erb M (2013) Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling. **New Phytol** 200: 1234–1246

Meldau S, Kästner J, Von Knorre D, Baldwin IT (2014) Salicylic acid-dependent gene expression is activated by locomotion mucus of different molluscan herbivores. **CommunIntegr Biol** 7: e28728

Oh Y, Baldwin IT, Gális I (2012) NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in *Nicotiana attenuata* plants. **Plant Physiol** 159: 769–788

Petersen C, Woods HA, Kingsolver JG (2000) Stage-specific effects of temperature and dietary protein on growth and survival of *Manduca sexta* caterpillars. **Physiol Entomol** 25: 35–40

Pinheiro J, Bates D, DebRoy S, Sarkar D, The R Development Core Team (2013) *Nlme: Linear and Nonlinear Mixed Effects Models*. R Package Version 3. pp. 1–111

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW, Jander G (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. **Plant Physiol** 158: 854–863

R Core Team (2012) R: A language and environment for statistical computing. URL http://www.R-project.org

Sanchez A, Shin J, Davis SJ (2011) Abiotic stress and the plant circadian clock. **Plant Signal Behav** 6: 223–231

Schittko U, Preston CA, Baldwin IT (2000) Eating the evidence? *Manduca sexta* larvae can not disrupt specific jasmonate induction in *Nicotiana attenuata* by rapid consumption. **Ecology** 49: 343–346

Schuman MC, Palmer-Young EP, Schmidt A, Gershenzon J, Baldwin IT (2014) Ectopic TPS expression enhances sesquiterpene emission in *Nicotiana attenuata* without altering defense or development of transgenic plants or neighbors. **Plant Physiol** 166: 779–797

Schwachtje J, Baldwin IT (2008) Why does herbivore attack reconfigureprimary metabolism? **Plant Physiol** 146: 845–851

Shin J, Heidrich K, Sanchez-Villarreal A, Parker JE, Davis SJ (2012) TIME FOR COFFEE represses accumulation of the MYC2 transcription factor to provide time-of-day regulation of jasmonate signaling in *Arabidopsis*. **Plant Cell** 24: 2470–2482

Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G (2006) XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching and identification. **Anal Chem** 78: 779–787

Steppuhn A, Baldwin IT (2008) Induced defenses and the cost-benefit paradigm. In: Schaller A, ed. *Induced Plant Resistance to Herbivory*. Springer Science + Business Media B. V.: Dordrercht, The Netherlands. pp. 61–83

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. **PLoS Biol** 2: e217

Sysoeva MI, Markovskaya EF, Shibaeva TG (2010) Plants under continuous light: A review. **Plant Stress** 4: 5–17

Tautenhahn R, Boettcher C, Neumann S (2008) Highly sensitive feature detection for high resolution LC/MS. **BMC Bioinfo** 9: 504

Wang W, Barnaby JY, Tada Y, Li H, To M, Fu X, Dong X (2011) Timing of plant immune responses by a central circadian regulator. **Nature** 470: 110–115

Wigglesworth VB (1972) *The Principles of Insect Physiology*. Chapman and Hall, New York, NY, USA

Wu J, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. **Ann Rev Genet** 44: 1–24

Wünsche H, Baldwin IT, Wu J (2011) Silencing NOA1 elevates herbivory-induced jasmonic acid accumulation and compromises most of the carbon-based defense metabolites in *Nicotiana attenuata*. **J Integr Plant Biol** 53: 619–631

Xia J, Sinelnikov I, Han B, Wishart DS (2015) MetaboAnalyst 3.0 – making metabolomics more meaningful. **Nucl Acids Res** 43: W251–W257 (DOI: 10.1093/nar/gkv380)

Xia J, Mandal R, Sinelnikov I, Broadhurst D, Wishart DS (2012) MetaboAnalyst 2.0 – a comprehensive server for metabolomic data analysis. **Nucl Acids Res** 40: W127–W133

Xia J, Psychogios N, Young N, Wishart DS (2009) MetaboAnalyst: A web server for metabolomic data analysis and interpretation. **Nucl Acids Res** 37: W652–W660

Yerushalmi S, Green RM (2009) Evidence for the adaptive significance of circadian rhythms. **Ecol Lett** 12: 970–981

Zavala JA, Baldwin IT (2004) Fitness benefits of trypsin proteinase inhibitor expression in *Nicotiana attenuata* are greater than their costs when plants are attacked. **BMC Ecol** 4: 11

Zhang C, Xie Q, Anderson RG, Ng G, Seitz NC, Peterson T, McClung CR, McDowell JM, Kong D, Kwak JM, Lu H (2013) Crosstalk between the circadian clock and innate immunity in *Arabidopsis*. **PLoS Pathog** 9: e1003370

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) *Mixed Effects Models and Extensions in Ecology with R*. In: Gail M, Krickeberg K, Samet JM, Tsiatis A, Wong W, eds. Springer Science + Business Media, LLC, New York, NY, USA

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Metabolic fingerprints from in-phase and out-of-phase *Nicotiana attenuata* plants become less similar after 1 d exposure to constant light or constant dark, but more similar after 3 d of herbivory

Principal component analyses (PCAs) were conducted on metabolomic fingerprints of plant samples ($n = 5$ plants/treatment). For constant conditions, samples were collected before transfer to constant light (LL, **A**) or constant dark (DD, **B**), as well as before and after *Manduca sexta* infestation. Dotted lines delineate 95% confidence intervals. Treatment groups were separated by the effects of induced defense in response to 3 d herbivory (PC1) as well as effects due to prior entrainment conditions and/or their interactions with exposure to 1 d of constant conditions (PC2)

Figure S2. Volcano plots showing individual m/z features demonstrate fewer differences between in-phase and out-of-phase plants after 3 d herbivory in LL and LD than in DD

The x-axis shows the $\log_2$-transformed fold change (FC) between in-phase and out-of-phase plants for the individual m/z features, and the y-axis shows the log10-transformed *P*-values for t-tests between in-phase and out-of-phase plants for the individual m/z feature; $n = 5$. Significant m/z features are denoted by $P < 0.05$ and a fold change ≤ -2 or ≥ 2 (cut-off values denoting threshold lines in the graphs)

Figure S3. Plants kept under constant dark display hyponasty and carbon starvation

Hyponasty (**A**) is indicative of a shade-avoidance response in mature plants. (**B**) Starch content was compared between in-phase and out-of-phase plants within a time point using independent sample t-tests ($n = 5$). Because in-phase plants were exposed to a 12 h night prior to DD treatment but out-of-phase plants were exposed to a 12 h day, out-of-phase plants initially had greater amounts of starch ($^{***}P < 0.001$ in a Student's *t*-test versus in-phase plants at 0 d), but in-phase and out-of-phase plants had similar starch levels after 1 d of acclimation.

Figure S4. Damage to *Nicotiana attenuata* leaves by second-instar *Manduca sexta* larvae is roughly constant per unit larval mass under constant light and temperature conditions, but fluctuates under diurnal conditions

Leaf area damage caused by five 2nd-instar *M. sexta* larvae (leaf area in cm^2 g larval $mass^{-1}$ h^{-1}) after 24 h acclimatization under the three experimental regimes used (see Figure 1), mean ± SE ($n = 5$–6). Note that Y-axes have different scales. Damage rates per unit larval mass were relatively constant under constant light (LL) conditions or constant dark (DD) conditions, but fluctuated under diurnal (LD) conditions; for choice of temperatures, see Results text and Table S3. ^{a,b}Damage per unit mass was significantly greater in the two 4 h time intervals from 13:00 to 21:00 than in all other 4 h time intervals under LD conditions: intervals which did not significantly differ were grouped and compared in a linear mixed effects model (likelihood ratio test, $P < 0.0001$).

Table S1. Results of Wilcoxon rank-sum tests comparing masses of larvae on in-phase versus out-of-phase plants; the significant *P*-value is in bold

Table S2. Results of Student's t-tests comparing concentrations of target metabolites from in-phase versus out-of-phase plants; significant *P*-values are in bold

Table S3. Mean temperatures (in °C) measured using temperature loggers at four different positions distributed in climate chambers used for *Manduca sexta* growth assays (Figure 2) over at least 2 d of measurements taken every 10 min

Table S4. Mean masses (in mg) of *Manduca sexta* larvae used for feeding damage

File S1. Significant features identified in DD, LL, and LD volcano plots (volcano.xlsx)

Supplemental materials and methods